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Involvement of *Rhodocyclus*-Related Organisms in Phosphorus Removal in Full-Scale Wastewater Treatment Plants

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The participation of organisms related to *Rhodocyclus* in full-scale enhanced biological phosphorus removal (EBPR) was investigated. By using fluorescent in situ hybridization techniques, the communities of *Rhodocyclus*-related organisms in two full-scale wastewater treatment plants were estimated to represent between 13 and 18% of the total bacterial population. However, the fractions of these communities that participated in polyphosphate accumulation depended on the type of treatment process evaluated. In a University of Cape Town EBPR process, the percentage of *Rhodocyclus*-related cells that contained polyphosphate was about 20% of the total bacterial population, but these cells represented as much as 73% of the polyphosphate-accumulating organisms (PAOs). In an aerated-anoxic EBPR process, *Rhodocyclus*-related PAOs were less numerous, accounting for 6% of the total bacterial population and 26% of the total PAO population. In addition, 16S ribosomal DNA sequences 99.9% similar to the sequences of *Rhodocyclus*-related organisms enriched in acetate-fed bench-scale EBPR reactors were recovered from both full-scale plants. These results confirmed the involvement of *Rhodocyclus*-related organisms in EBPR and demonstrated their importance in full-scale processes. In addition, the results revealed a significant correlation between the type of EBPR process and the PAO community.

Phosphorus removal from wastewater can be accomplished biologically in activated sludge reactors by incorporating an anaerobic stage prior to existing aerobic basins (10). The resulting cyclic anaerobic and aerobic conditions favor the growth of microorganisms that utilize intracellular polyphosphate as an energy source during the anaerobic period, which allows them to sequester available carbon for use during the following aerobic stage (18). In turn, the aerobic utilization of intracellular stored carbon is accompanied by the uptake of phosphorus and accumulation of phosphorus as polyphosphate. This polyphosphate accumulation results in efficient removal of phosphorus from the wastewater. Although this process, termed enhanced biological phosphorus removal (EBPR), has been used successfully in full-scale wastewater treatment plants (WWTPs), identification and characterization of the industrially relevant organisms that are involved in phosphate uptake have proven to be difficult (18). In the initial attempts to identify polyphosphate-accumulating organisms (PAOs) the workers used enrichment cultures and traditional culturing approaches (5, 9, 16, 25). However, the microorganisms recovered (predominantly *Acinetobacter* sp.) did not exhibit all the biochemical characteristics believed to be required for cyclic phosphate uptake and release and were later shown, by a variety of culture-independent methods, to be minor components of the microbial communities in full-scale EBPR processes (4, 6, 12, 13, 23). Recently, working with acetate-fed laboratory-scale reactors, Hesselmann et al. (11) and Crocetti et al. (7) provided evidence that when an organism closely related to *Rhodocyclus* (proposed name, *Candidatus* Accumulibacter phosphatis [11]) is enriched in cyclic anaerobic-aerobic conditions, efficient phosphorus removal is achieved. To date, no pure cultures of this organism have been reported, and its importance in full-scale EBPR processes has not been assessed.

The work presented here was designed to test the relevance of the *Rhodocyclus*-related organism identified by Hesselmann et al. (11) and Crocetti et al. (7) for phosphorus removal in full-scale WWTPs. Activated sludge samples from three full-scale WWTPs were analyzed by fluorescent in situ hybridization (FISH) targeting activated sludge samples and PAO-rich subsamples obtained by physically separating PAOs from other microorganisms present in activated sludge. In addition, the *Rhodocyclus*-related organisms in the three treatment plants were evaluated by comparative analysis of partial 16S ribosomal DNA (rDNA) sequences recovered from the activated sludge samples.

MATERIALS AND METHODS

Activated sludge samples. Activated sludge samples were collected from three full-scale WWTPs with distinct operational conditions. The Nine Springs WWTP (158,000 m^3 day^{-1}) in Madison, Wis., operated as a University of Cape Town process plant without nitrate recycling, represented a traditional EBPR process. The Dane-Iowa treatment plant (2,200 m^3 day^{-1}) in Mazomanie, Wis., is an aerated-anoxic Orbal process plant achieving simultaneous nitrification-denitrification and biological phosphorus removal. The Racine WWTP (108,000 m^3

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TABLE 1. Oligonucleotide probes used for FISH

Probe	Specificity	Sequence (5′-3′)	% Formamide	Reference(s)
EUB338	Bacteria	GCTGCCTCCCGTAGGAGT	20	2
ALF1b	α-*Proteobacteria*	CGTTCG(C/T)TCTGAGCCAG	20	17
BET42a	β-*Proteobacteria*	GCCTTCCCACTTCGTTT	35	17
GAM42a	γ-*Proteobacteria*	GCCTTCCCACATCGTTT	35	17
HGC	Gram positive, high G+C content	TATAGTTACCACCGCCGT	25	23
CF	*Cytophaga-Flavobacterium*	TGGTCCGTGTCTCAGTAC	35	23
RHC439	*R. tenuis* subgroup	CNATTTCTTCCCCGCCGA	30[a]	11
PAO462b	*R. tenuis* subgroup	CCGTCATCTRCWCAGGGTATTAAC	35	7; this study
PAO651	*R. tenuis* subgroup	CCCTCTGCCAAACTCCAG	35	7
PAO846b	*R. tenuis* subgroup	GTTAGCTACGGYACTAAAAGG	35	7; this study

[a] When the four probes targeting the *R. tenuis* subgroup were used simultaneously, 35% formamide was used.

day^{-1}) in Racine, Wis., is a conventional process plant with phosphorus removal by chemical precipitation and represented an activated sludge reactor without EBPR. All the plants treated municipal wastewater with phosphorus concentrations of 7 to 8 mg of P $liter^{-1}$ and achieved effluent concentrations of less than 1 mg of P $liter^{-1}$.

Microscopic analysis. Grab samples of activated sludge were collected from the end of the aerobic stage of each full-scale treatment plant and were stored on ice during transport to the laboratory. Dispersion of flocs and cell fixation were performed within 2 h of sampling. Settled mixed liquor samples were mechanically disrupted by repetitive purging through a 26-gauge needle at least 30 times. Samples were then fixed in 3% paraformaldehyde in phosphate-buffered saline (130 mM NaCl, 10 mM Na_2HPO_4; pH 7.2) for 30 min at room temperature. Following fixation, cells were washed to remove the fixative, collected by filtration with a 0.2-μm-pore-size polycarbonate filter, and transferred to gelatin-coated slides (2). Polyphosphate staining was performed for 1 h with a 1-μg ml^{-1} DAPI (4′,6′-diamidino-2-phenylindole) solution (13a, 26).

FISH was performed by using established protocols (17). Table 1 summarizes the oligonucleotide probes used in this study, along with the hybridization conditions and related references. Unlabeled BET42a and GAM42a were used as competitors for each other as previously described (24). For hybridization of *Rhodocyclus*-related organisms, a combination of four probes (RHC439, PAO462b, PAO651, and PAO846b) was used to increase the fluorescent response, as previously reported (7). The *Rhodocyclus*-specific probes PAO462b and PAO846b were derived from the original PAO462 and PAO846 probes designed by Crocetti et al. (7) by introducing a degeneracy that allowed inclusion of two *Rhodocyclus*-related clones previously retrieved from the Nine Springs WWTP (UCT N123 and UCT N141 in Fig. 2). Cells were visualized with a Zeiss Axioplan 2 epifluorescent microscope (Carl Zeiss, Thornwood, N.Y.). A typical microscopic analysis consisted of manually counting fluorescently labeled cells in at least 10 photographs per well for duplicate wells, which resulted in total counts of 500 to 1,500 cells per sample. The 95% confidence intervals were typically ±5 to 10%.

Phosphate release experiments. Phosphate release from activated sludge was measured in anaerobic batch tests by using 2-liter portions of activated sludge samples collected at the end of the aerobic stage, incubated for at least 30 min for consumption of dissolved oxygen, and then spiked with sodium acetate to obtain an initial acetate concentration of 1.0 to 1.8 mM. Acetate concentrations were measured by gas chromatography with a Perkin-Elmer 3920 gas chromatograph and a Carbopack C/0.3% Carbowax 20 M/0.1% H_3PO_4 column (30 by 0.25 in.), and phosphate concentrations were measured by modified ascorbic acid method 4500-P (3).

Physical separation of PAOs. Separation of PAOs from non-PAOs was achieved by two different methods by using previously described protocols (Hung et al., submitted.). The first method relied on the higher buoyant density of some PAOs than of other organisms in the activated sludge samples. Briefly, aliquots of mechanically dispersed (26-gauge needle, 30 passes) sludge were added to centrifuge tubes containing a 20% Percoll colloidal suspension (Pharmacia, Uppsala, Sweden) and centrifuged for 60 min at 32,000 × *g* to establish a density gradient. With this procedure, PAOs accumulated at the bottom of each of the centrifuge tubes, while the majority of the other cells were concentrated towards the centers of the tubes (Hung et al., submitted.).

The second separation method was based on the differential staining of PAOs and non-PAOs with DAPI (Hung et al., submitted.). Activated sludge samples were fixed and dispersed as described above, and they were stained with DAPI (final concentration, 5 μg ml^{-1}) overnight on ice. Flow cytometry was performed with a FACStar Plus dual-laser flow cytometer (Becton Dickinson, San Jose, Calif.). DAPI was excited with multiline UV light generated with a krypton laser, and emission signals were collected through a 450/50 band pass filter and a 575/25 band pass filter. In this analysis, DAPI-stained PAOs have a high 575/450 fluorescence ratio, a characteristic that can be used to sort out the PAOs and obtain a PAO-rich subpopulation (26; Hung et al., submitted.).

Clone libraries. Two different clone libraries were obtained from the activated sludge samples analyzed. Sequences of 16S rRNA genes from organisms related to *Rhodocyclus* were retrieved by PCR amplification by using RHC439f (5′-TC GGCGGGGAAGAAATNG-3′) (11) and universal primer 1492r (5′-GGYTAC CTTGTTACGACTT-3′) (14). Each reaction tube contained 50 μl of 1× PCR buffer B, 2.5 U of *Taq* polymerase (Promega, Madison, Wis.), each primer at a concentration of 0.4 μM, each deoxynucleoside triphosphate at a concentration of 0.2 mM, 2.5 mM $MgCl_2$, 100 μg of bovine serum albumin ml^{-1}, and ~100 ng of genomic DNA. *Taq* polymerase was added after a 5-min hot start at 80°C; this was followed by an initial denaturation step at 94°C and 30 cycles of denaturation (90 s at 94°C), annealing (60 s at 65°C), and extension (180 s at 72°C) and then a final extension step at 72°C for 7 min. The temperature cycle was optimized for amplification of sequences without mismatches compared to RHC439f by using clones UTC N032 and UTC N173, which had one mismatch and no mismatch compared to RHC439f, respectively. Amplification products were verified by gel electrophoresis and were purified by using a Qiaex II gel extraction kit (Qiagen, Valencia, Calif.). Purified fragments were ligated to the pGEM-T vector (Promega) and transformed into *Escherichia coli* strain JM109 competent cells. Plasmids were extracted by using the Wizard *Plus* miniprep DNA purification system (Promega). Plasmid inserts were cycle sequenced with a Big Dye sequencing kit (Applied Biosystems, Foster City, Calif.) by using T7 and SP6 primers and approximately 1 μg of plasmid template. Products were purified by using Autoseq G-50 columns (Amersham Pharmacia Biotech Inc., Piscataway, N.J.) and then separated and analyzed with an automated DNA sequencer (ABI model 377XL; Applied Biosystems).

Additional information for *Rhodocyclus*-related PAOs in the Nine Springs treatment plant was obtained by PCR amplification of 16S rDNA fragments from a PAO-rich subpopulation obtained by density centrifugation. In this case, DNA was extracted from cells that accumulated at the bottom of each of the centrifuge tubes and was purified from LMP agarose gels (Promega) by using Wizard PCR preps (Promega). Amplification was performed with the 27f and 1492r universal primers (14). The thermocycler conditions included an initial denaturation step of 5 min at 94°C, 30 cycles of 1 min at 94°C, 2 min at 52°C, and 3 min at 62°C, and 30 min of incubation at 72°C. Following amplification, samples were again verified and purified from LMP agarose gels (Promega) by using Wizard PCR preps (Promega). The subsequent steps were identical to those described above for the *Rhodocyclus*-specific libraries, except that putative clones were initially screened by amplification with the T7 and SP6 primers, followed by gel electrophoresis. The amplified inserts were also used for restriction fragment length polymorphism analysis with the *Hae*III restriction enzyme (Amersham Pharmacia Biotech Inc.).

Retrieved sequences were compared with available 16S rDNA sequences by using BLAST (1). Sequences were aligned with ClustalX software (22). The Phylip software package was used for construction of phylogenetic trees by the neighbor-joining method and for construction of similarity matrixes (8, 21).

Nucleotide sequence accession numbers. The nucleotide sequences determined in this study have been deposited in the GenBank database under accession numbers AY062125, AY062126, AF450455 to AF450477, and AY064176 to AY064192.

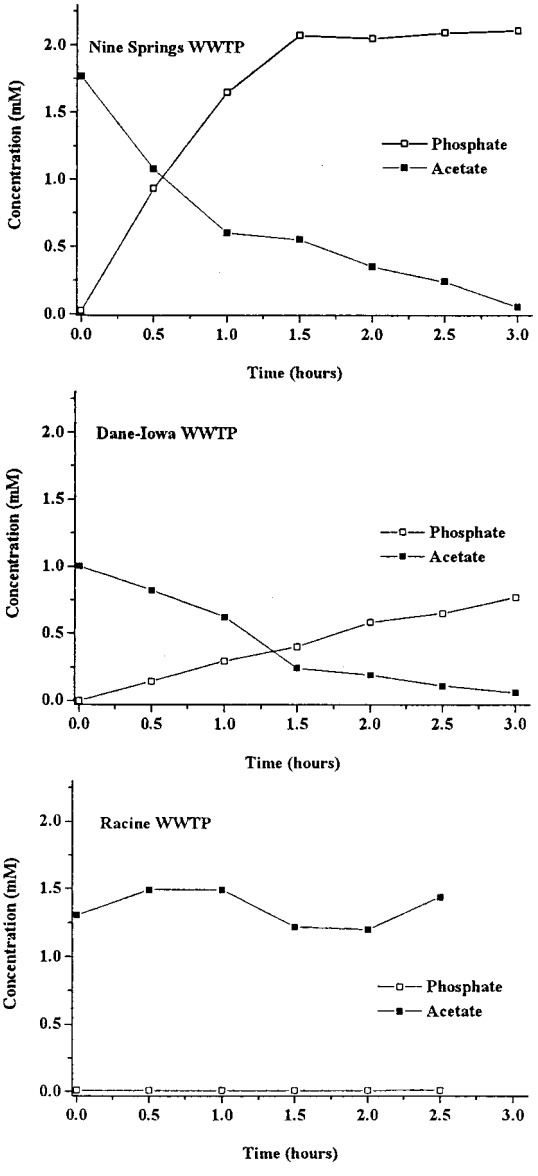

FIG. 1. Release of phosphorus (□) and uptake of acetate (■) during anaerobic batch tests of activated sludge from full-scale WWTPs.

RESULTS AND DISCUSSION

Two full-scale EBPR reactors (Nine Springs and Dane-Iowa WWTPs) and one full-scale chemical precipitation reactor (Racine WWTP) were included in this study. The EBPR activity within each reactor was evaluated by comparing the rates of phosphate release from an activated sludge sample maintained under anaerobic conditions and spiked with acetate. As shown in Fig. 1, the samples from the Nine Springs and Dane-Iowa WWTPs showed the acetate uptake and phosphorus release expected for an EBPR process. Neither phosphorus release nor acetate uptake was observed with the sludge from the Racine WWTP, consistent with this plant's operation as a conventional process with phosphorus removal by chemical precipitation.

Activated sludge samples from the three reactors were examined by using FISH with a combination of four oligonucleotide probes (RHC439, PAO462b, PAO651, and PAO846b) targeting *Rhodocyclus*-related organisms (Table 1). Both EBPR plants had significant numbers of hybridized cells (referred to as *Rhodocyclus*-positive cells below), which represented 18% ± 6% and 13% ± 7% of the total numbers of cells in the samples from the Nine Springs and Dane-Iowa plants, respectively. *Rhodocyclus*-positive cells were also detected in activated sludge samples from the Racine treatment plant, but their low numbers and the highly variable background fluorescence in these samples precluded quantification. The presence of a significant number of *Rhodocyclus*-positive cells in the two EBPR processes suggested that the laboratory studies of Hesselmann et al. (11) and Crocetti et al. (7) had indeed identified an organism that was relevant in full-scale EBPR. To further assess the level of involvement of the organism in phosphorus removal, correlation between polyphosphate accumulation and phylogenetic identification was pursued.

Physical separation and analysis of PAOs. By simultaneously performing DAPI staining of intracellular polyphosphate granules and FISH (26), it was possible to visualize some *Rhodocyclus*-positive cells that contained polyphosphate granules in samples from the two EBPR process WWTPs studied, which confirmed the involvement of these organisms in phosphorus removal. However, when the numbers of PAO in DAPI-stained and DAPI-stained FISH samples were compared, it was evident that the number of polyphosphate-positive cells decreased when the samples were subjected to the hybridization and washing steps of the FISH protocol (19, 26). Therefore, to obtain meaningful quantification of the number of *Rhodocyclus*-positive cells that contained polyphosphate, we developed two procedures to physically separate PAO from other organisms in activated sludge. After the physical separation, the resulting PAO-rich subpopulations were used for further analyses.

Initially, PAO separation and concentration were accomplished based on differences in buoyant density between PAOs and non-PAOs. When activated sludge samples from the Nine Spring WWTP were subjected to density gradient separation, a large number of PAOs were concentrated in the bottoms of centrifuge tubes, while the majority of the cells in the samples clustered in the middle of the tubes (Hung et al., submitted.). The density centrifugation method produced subpopulations containing as much as 43% PAOs. The presence of *Rhodocyclus*-related organisms in these PAO-rich subpopulations was evaluated by FISH and by comparative 16S rDNA sequence analyses. These analyses revealed that *Rhodocyclus*-related organisms preferentially accumulated in the bottoms of the centrifuge tubes, a result that was consistent with the importance of *Rhodocyclus*-related PAOs in the full-scale EBPR process.

The PAO-rich subsample obtained by density centrifugation was also examined for the presence of *Rhodocyclus*-related organisms by creating a clone library of nearly complete 16S rDNA fragments by performing PCR amplification with universal primers. Of a total of 70 unique clones retrieved and sequenced, 6 were closely related to the *Rhodocyclus tenuis* subgroup of the β subclass of the *Proteobacteria* (β-*Proteobacteria*) (designated UCT N clones). A sequence similarity evaluation indicated that three of the retrieved clones (UCT N112,

TABLE 2. Taxonomic analyses of the nonsorted and sorted samples from Nine Springs and Dane-Iowa WWTPs

Targeted group	% of total cells in:			
	Nine Springs activated sludge	Nine Springs PAO-rich subsample	Dane-Iowa activated sludge	Dane-Iowa PAO-rich subsample
PAO	28[b]	58[d]	22[b]	52[d]
Bacteria	78	77	83	50
α-*Proteobacteria*	10	9	4	7
β-*Proteobacteria*	25	58	12	5
γ-*Proteobacteria*	3	5	7	10
Gram positive, high G+C content	9	8	15	11
Cytophaga-Flavobacterium	ND[e]	13	4	5
R. tenuis subgroup	18[a]	41[c]	13[a]	18[c]

[a] $Rhcy^+$ values used in equation 2.
[b] $PolyP^+$ values used in equation 3.
[c] $(Rhcy^+)_{conc}$ values used in equation 5.
[d] $(PolyP^+)_{conc}$ values used in equation 6.
[e] ND, not determined.

UCT N161, and UCT N173) were >98% similar to the clone used as the basis for the proposal of the new genus and species *Candidatus* A. phosphatis made by Hesselmann et al. (11), suggesting that *Candidatus* A. phosphatis is present in full-scale EBPR processes.

An independent approach to physically separate PAOs from non-PAOs was employed to focus further phylogenetic analyses on PAOs. As described elsewhere (26; Hung et al., submitted.), DAPI can be used to differentially stain PAOs and allow efficient separation by flow cytometry. When applied to activated sludge samples from the full-scale EBPR reactors, this method produced subpopulations with 58 and 52% PAOs for the Nine Springs and Dane-Iowa WWTPs, respectively. For the Racine WWTP, it was not possible to recover a PAO-rich subpopulation, as differential staining did not reveal any significant concentration of PAOs in the samples.

The PAO-rich subsamples obtained by flow cytometry were evaluated by FISH by using probes for taxonomically defined groups, as well as the set of probes for *Rhodocyclus*-related bacteria (Table 2). For the Nine Springs samples, concentration of *Rhodocyclus*-positive cells and members of the β-*Proteobacteria* was evident in the flow cytometry PAO-rich subsamples, while other taxonomic groups were not significantly concentrated. This result was similar to that obtained when PAOs were concentrated by density centrifugation and provided independent validation of the involvement of *Rhodocyclus*-positive cells in phosphate removal at this full-scale EBPR plant. In contrast, the sorted samples obtained from the Dane-Iowa WWTP showed a smaller increase in *Rhodocyclus*-positive cells (i.e., from 13 to 18%) and did not result in concentration of any of the broad taxonomic groups analyzed. These results suggested that the *Rhodocyclus*-positive population was less important for phosphorus removal in the Dane-Iowa WWTP. Furthermore, since none of the taxonomic groups were significantly concentrated while PAOs were concentrated from 22 to 52%, the polyphosphate-accumulating activity at the Dane-Iowa WWTP may either be distributed among a diverse group of organisms or be concentrated in organisms not targeted by the probes used in this study.

Quantification of polyphosphate-positive and *Rhodocyclus*-positive cells. The FISH analyses of activated sludge and PAO-rich subpopulations described above provided evidence that polyphosphate is accumulated by organisms related to *Rhodocyclus.* These analyses, however, did not provide direct quantification of the fraction of *Rhodocyclus*-positive cells that were actively involved in phosphorus removal. Such quantification is important for overall assessment of the involvement of *Rhodocyclus*-related organisms in the EBPR process, especially since *Rhodocyclus*-positive, polyphosphate-negative cells have been reported (26).

Indirect evaluation of the relative number of cells that were simultaneously polyphosphate positive and *Rhodocyclus* positive was accomplished by comparing the individual populations with these two characteristics in the activated sludge and PAO-rich samples. If the bacterial community in the activated sludge is broadly defined as having four distinct groups, namely, *Rhodocyclus*-positive and polyphosphate-positive cells (A), *Rhodocyclus*-positive and polyphosphate-negative cells (B), *Rhodocyclus*-negative and polyphosphate-positive cells (C), and *Rhodocyclus*-negative and polyphosphate-negative cells (D), then the mass balances for total cells, *Rhodocyclus*-positive cells ($Rhcy^+$), and polyphosphate-positive cells ($PolyP^+$) could be represented by equations 1 through 3, respectively; microscopic analyses of FISH data and DAPI-stained activated sludge provided experimental estimates of the *Rhodocyclus*-positive cells and the polyphosphate-positive cells (Table 2).

$$A + B + C + D = 100\% \quad (1)$$

$$A + B = Rhcy^+ \quad (2)$$

$$A + C = PolyP^+ \quad (3)$$

Furthermore, the PAO-rich subpopulations obtained by flow cytometry provide additional information concerning the composition of the activated sludge community. If n and m represent the concentrations of cells with and without polyphosphate in a PAO-rich subpopulation, respectively, and the concentration factor is independent of whether a cell is *Rhodocyclus* positive, then equations 4 through 6 can be used to describe equivalent mass balances in the PAO-rich samples; estimates of $(Rhcy^+)_{conc}$ and $(PolyP^+)_{conc}$ were obtained from independent quantifications of *Rhodocyclus*-positive and polyphosphate-positive cells in the PAO-rich subsamples (Table 2).

$$nA + mB + nC + mD = 100\% \quad (4)$$

$$nA + mB = (Rhcy^+)_{conc} \quad (5)$$

$$nA + nC = (PolyP^+)_{conc} \quad (6)$$

Equations 1 through 6 form a system of six independent equations with six unknowns that can be solved for the Nine Springs and Dane-Iowa samples by using the data presented in Table 2. Accordingly, for the Nine Springs samples, the percentage of cells that were simultaneously *Rhodocyclus* positive and polyphosphate positive was estimated to be 20%. Since the total PAO population at Nine Springs was 28% of the total population, then *Rhodocyclus*-related cells accounted for 73% of the total PAO population. This calculation suggested that a significant fraction of the phosphate removal activity at Nine

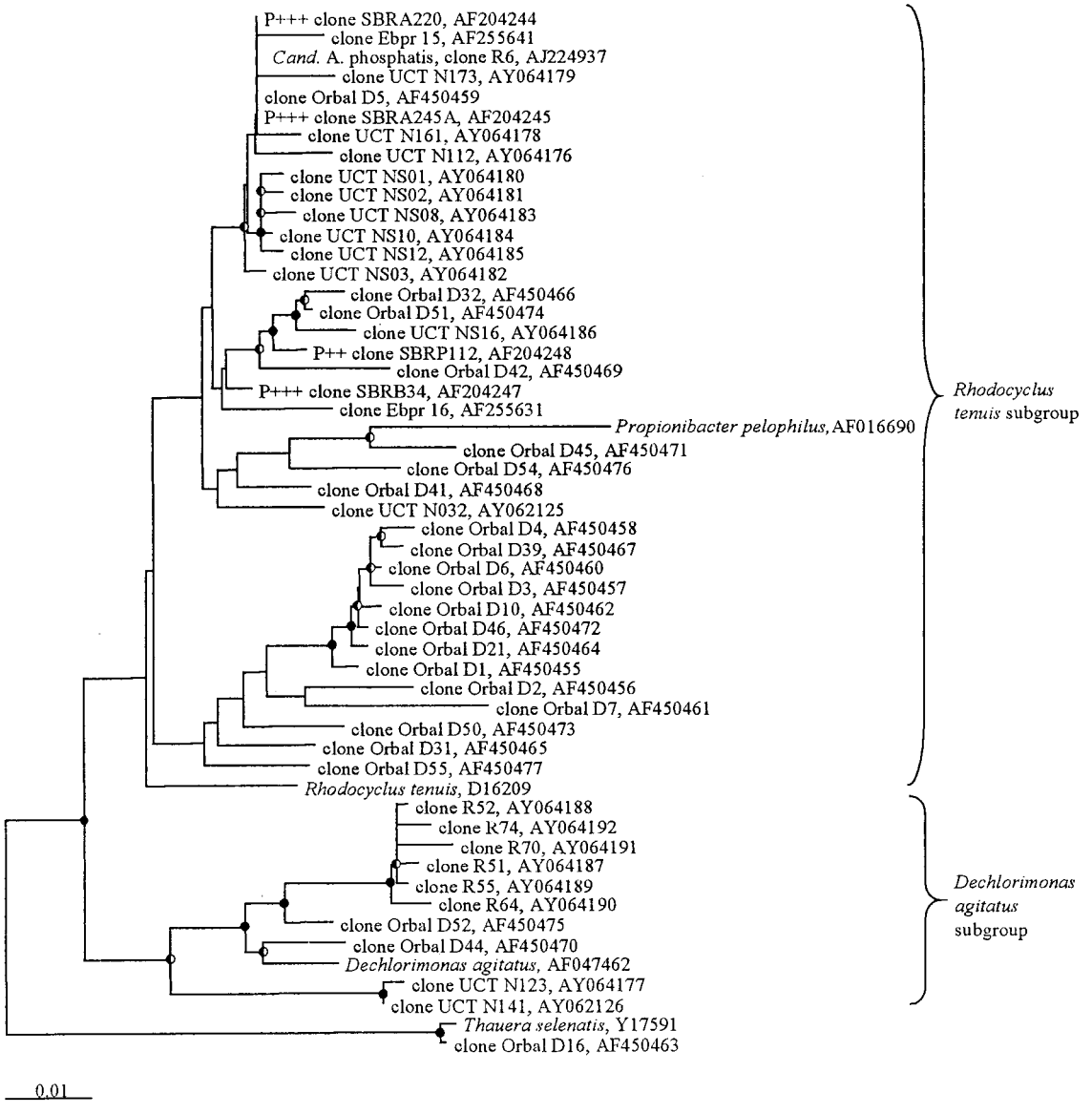

FIG. 2. Phylogenetic tree for partial 16S rDNA sequences from *Rhodocyclus*-related clones retrieved from activated sludge of full-scale WWTPs obtained by using universal primers (UCT N clones from Nine Springs WWTP) or primers RHC439f and 1492r (UCT NS clones from Nine Springs WWTP; Orbal D clones from Dane-Iowa WWTP; R clones from Racine WWTP) along with related sequences retrieved from GenBank. Scale bar = 0.01 substitution per nucleotide position. The tree was rooted by using the 16S rDNA sequence from *Rubrivivax gelatinosus* D16213 (data not shown) as an outgroup. Solid circles at nodes indicate >75% bootstrap support, while half-solid circles indicate 50 to 75% bootstrap support based on 1,000 resamplings.

Springs is catalyzed by *Rhodocyclus*-related organisms. Moreover, the proportion of *Rhodocyclus*-related organisms in the Nine Springs activated sludge was 18% ± 6%, and thus, this analysis suggests that all the *Rhodocyclus*-positive cells in this sludge were actively participating in the EBPR process. Although some *Rhodocyclus*-positive, polyphosphate-negative cells have been observed in Nine Springs samples (26), our analysis suggests that this population is a minor component of the microbial community. For the Dane-Iowa WWTP, the calculated percentage of *Rhodocyclus*-positive and polyphosphate-positive cells (as determined by using equations 1 through 6 and the data in Table 2) was 6%. With a total PAO percentage in this treatment plant of 22%, *Rhodocyclus*-related cells represented 26% of the total PAO community, suggesting that the involvement of these organisms in phosphorus removal activity is less significant at Dane-Iowa than at Nine Springs. In addition, since the measured percentage of *Rhodocyclus*-related cells in the Dane-Iowa WWTP was 13% ± 7%, about one-half of the cells identified as *Rhodocyclus*-positive cells did not contain polyphosphate and were probably not involved in the EBPR process. For the Racine samples, a similar analysis was not possible because a PAO-rich subpopulation was not observed and because these samples exhibited high background fluorescence in FISH experiments. Neverthe-

TABLE 3. Mismatch information for oligonucleotide probes and recovered 16S rDNA clones

Subgroup	Clone(s)	No. of mismatches			
		RHC439[a]	PAO462b	PAO651	PAO846b
R. tenuis subgroup	*Candidatus* A. phosphatis SBRA220, Ebpr 15, UCT N173, Orbal D5, SBRA245A, UCT N161, UCT N112	0	0	0–1	0
	Six UCT NS clones	0	0–1	0	0
	Orbal D32, Orbal D51, UCT NS16, SBRP112, Orbal D42, SBRB34, Ebpr 16	0–1	0–3	0–2	0
	Orbal D41	0	1	2	2
	UCT N032	1	1	0	0
	Nine D clones	0	6–7	1–2	0–2
	Orbal D7	0	7	3	2
	Orbal D50	0	2	2	2
	Orbal D31, Orbal D55	0	0	0–1	2
	P. pelophilus, Orbal D45, Orbal D54	0–2	6–8	0–3	2
	R. tenuis	0	4	1	2
D. agitatus subgroup	Six R clones	0	1	3	2
	Orbal D52	0	2	3	1
	D. agitatus, Orbal D44 UCT N123, UCT N141	0–1	4–6	3	1–3
		3	0	0–3	0
Thauera aromatica subgroup	*Thauera selenatis*, Orbal D16	0–1	11	3	5–6

[a] Clones retrieved with the RHC439f primer (NS, D, and or R clones) were presumed to have zero mismatches.

less, the inability to obtain a PAO-rich subpopulation is consistent with this plant removing phosphorus by chemical precipitation instead of EBPR.

The differences in the enrichment of *Rhodocyclus*-related organisms in the three WWTPs probably reflect operational differences. Operation of the Nine Springs WWTP as a University of Cape Town process without nitrate recycling optimizes the separation of anaerobic, anoxic, and aerobic stages in the reactor. In contrast, the Dane-Iowa WWTP operates as an aerated-anoxic process plant in which simultaneous nitrification and denitrification are promoted and a strictly anaerobic environment is not observed (20). Under these circumstances, competition between PAO and denitrifying bacteria for volatile fatty acids might create less favorable conditions for enrichment of *Rhodocyclus*-related bacteria and favor enrichment of PAO not related to *Rhodocyclus*. Other important operational parameters that might result in differential enrichment of *Rhodocyclus*-related organisms in the two EBPR processes are the solids retention time (9 days for Nine Springs and 20 days for Dane-Iowa) and the significantly longer phosphate release stage in the Dane-Iowa process (11 h of aerated-anoxic stage) than in the Nine Springs WWTP (1.8 h of anaerobic stage). The failure to detect sequences related to *Candidatus* A. phosphatis in the non-EBPR Racine WWTP likely reflects the absence of cyclic anaerobic-aerobic conditions in this process.

***Rhodocyclus*-positive sequences from full-scale WWTPs.** In order to evaluate the diversity of microorganisms that hybridized with the set of *Rhodocyclus*-related probes used in this study, a clone library of 16S rRNA genes related to *Rhodocyclus* was constructed from DNA extracted from activated sludge samples from all three WWTPs. The phylogenetic relationship of the recovered clones to *Candidatus* A. phosphatis and other *Rhodocyclus*-related organisms is summarized in Fig. 2. Several sequences recovered from the two full-scale EBPR processes (UCT NS clones retrieved from the Nine Springs WWTP and Orbal D clones retrieved from the Dane-Iowa WWTP) clustered with *Candidatus* A. phosphatis and related clones previously retrieved from bench-scale acetate-fed reactors (7, 11, 15), supporting the industrial importance of these organisms. However, significant differences were also observed between the two EBPR processes. While the majority of the clones from Nine Springs clustered with *Candidatus* A. phosphatis, the majority of the clones from Dane-Iowa formed an independent branch, with similarities of <97% with other sequences shown in Fig. 2. This result is consistent with the observations and calculations based on FISH, which indicated that organisms related to *Rhodocyclus* were more important for phosphorus removal in the Nine Springs WWTP. Furthermore, these results suggested that the *Rhodocyclus*-positive, polyphosphate-negative organisms observed in Dane-Iowa samples might not be in the *Candidatus* A. phosphatis cluster. Finally, when the same protocol was applied to activated sludge samples from the non-EBPR Racine WWTP, all of the recovered clones (R clones) were associated with *Dechlorimonas agitatus* rather than with *Candidatus* A. phosphatis.

The *Rhodocyclus*-related clones recovered from each treatment plant are also useful for assessing the specificity of the set of probes used in the FISH analysis. The stringency of the in situ hybridization analyses conducted in this study was determined by using the formamide concentrations recommended by Crocetti et al. (7). Due to the unavailability of pure cultures, these authors estimated the optimal stringency for their enriched cultures by comparing the morphologies of the hybridized cells at different levels of formamide and selecting a formamide concentration that allowed hybridization only to a single morphotype (7). Because pure cultures are still unavailable, it is difficult to accurately assess the specificity of the hybridization of the four probes to the activated sludge from the full-scale processes. However, the additional sequences recovered here from full-scale WWTPs provided a larger data set for analyzing probe specificity. For probe RHC439, it is

likely that all the recovered clones corresponded to cells that hybridized to this probe, as RHC439f was used as the forward primer in the PCR amplification procedure and the PCR conditions were optimized by using clones with zero and one mismatches. As the amplified region contained the target sites for the other three probes used, the number of mismatches of these probes compared to the sequences of the organisms represented by the recovered clones was determined. A summary of this analysis is presented in Table 3. If the conservative assumption that probes with zero to two mismatches partially contributed to the observed hybridization intensity was made, all the representatives of *Candidatus* A. phosphatis and the UCT NS cluster were likely quantified by FISH. On the other hand, the independent cluster of Dane-Iowa clones and the sequences that clustered with *Propionibacter pelophilus* or *D. agitatus* had at least two mismatches with at least two of the four probes, and therefore the contributions of these sequences to the quantification of *Rhodocyclus*-positive cells during FISH was probably minimal. The recovered clones Orbal D41, Orbal D31, and Orbal D55 had lower numbers of mismatches and probably contributed to the Dane-Iowa *Rhodocyclus*-positive population, despite having only 95 to 97% overall similarity with the *Candidatus* A. phosphatis cluster. All clones recovered from the Racine WWTP had one, three, and two mismatches with probes PAO462b, PAO651, and PAO846b, respectively, which probably resulted in weak fluorescent signals and contributed to the difficulty in quantifying *Rhodocyclus*-positive cells in the Racine samples.

In summary, organisms related to *Rhodocyclus* represented significant fractions of the total bacterial communities in two full-scale EBPR processes. Based on analysis of samples physically enriched for PAO, *Rhodocyclus*-positive cells were estimated to account for 26 and 73% of the PAO in these two processes. In a non-EBPR plant, *Rhodocyclus*-positive cells and 16S rDNA sequences related to *Candidatus* A. phosphatis were not significant. Together, these results demonstrate that organisms related to *Rhodocyclus* and to *Candidatus* A. phosphatis are important in the phosphorus removal activity in full-scale EBPR processes and that indeed, the anaerobic-aerobic cyclic conditions in EBPR processes provide a selective advantage for enrichment of the population of these organisms in activated sludge.

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